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Award Number: W81XWH-10-1-0422

TITLE: Targeting Cell Surface Proteins in Molecular Photoacoustic Imaging to Detect Ovarian Cancer Early

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REPORT DATE: July 2012

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
July 2012	Annual	1 July 2011 - 30 June 2012

4. TITLE AND SUBTITLE
Targeting Cell Surface Proteins in Molecular Photoacoustic Imaging to Detect Ovarian Cancer Early

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-10-1-0422

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Fred Hutchinson Cancer Research Center
Seattle, WA 96109

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Our idea is to apply a series of novel techniques to identify the reagents needed to move imaging technology forward into the clinic. While molecular imaging strategies are now approaching the resolution required to detect ovarian cancer in an early curable stage, specific imaging probes are not currently available and are urgently needed to realize the potential of imaging for ovarian cancer early detection. To address this challenge we are undertaking a comprehensive proteomic analysis of the cell surface membrane of ovarian cancer cells. We have completed a survey of the cell surface glycoproteome of OVCAR3 cells and serous ovarian cancer cells isolated from ascites using a novel biochemical labeling method that allows for highly selective capture and internal validation of candidate peptides and proteins by LC-MS/MS. To date 411 cell surface proteins have been identified. We are currently annotating these proteins as potential targets for molecular imaging probes evaluating them by IHC. Several promising candidates have been identified.

15. SUBJECT TERMS
Ovarian Cancer, molecular imaging

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	7	19b. TELEPHONE NUMBER *(include area code)*

Targeting Cell Surface Proteins in Molecular Photoacoustic Imaging to Detect Ovarian Cancer Early

Charles W Drescher, MD, Principle Investigator

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INTRODUCTION

Our idea is to apply a series of novel techniques to identify the reagents needed to move imaging technology forward into the clinic. While molecular imaging strategies are now approaching the resolution required to detect ovarian cancer in an early, curable stage, specific imaging probes are not currently available and are urgently needed to realize the potential of imaging for ovarian cancer early detection. To address this challenge we are conducting for the first time a comprehensive, comparative survey of the surface proteome of serous ovarian cancer and human ovarian surface epithelial cells in order to select and validate ovarian cancer specific surface proteins for use as targets in molecular photoaccoustic imaging, an especially promising imaging strategy for ovarian cancer early detection.

BODY

Last year we reported on our experience using a novel hydrazine chemistry method for capturing N-linked glycoproteins from the surface of intact viable OVCAR3 cells. The approach proved highly successful with 209 candidate cell surface proteins identified. Here we report progress in identifying additional ovarian cancer cell surface proteins by applying the method to serous ovarian cancer cells isolated from ascites and also in prioritizing, ranking and validating our candidates for use a targets for ovarian cancer molecular imaging probes.

Aim 1) Identify ovarian cancer associated surface proteins from the set of proteins detected in a comparative LC-MS/MS-based analysis of the surface proteome of serous ovarian cancer and human ovarian surface epithelial cells.

Over the past year, we have used the N-linked glycoprotein capture method to profile ovarian cancer cells isolated from fresh ascites fluid and have compared the results to those found previously through profiling the ovarian cancer cell line OVCAR3. Roughly 85% of all proteins detected were subsequently confirmed by bioinformatic and experimental methods to be likely associated with the surface membrane.

The following features were used to annotate proteins as being derived from the cell surface: a) the presence of a N~XS/T motif, b) cell membrane localization as defined by the Gene Ontology (GO) Cellular Component database, c) the presence of transmembrane helices as determined by the TMHMM prediction program (http://www.cbs.dtu.dk/services/TMHMM/) and d) experimental evidence supporting a cell surface origin identified by review of Pubmed, Genecards and Information Hyperlinked over Proteins (www.ihop-net.org/UniPub/iHOP/) databases. GO defined cellular components for each protein sequence were determined using the generic GO slim (version 1.2) from the GO consortium (http://www.geneontology.org/GO.slims.shtml). GO slim files are reduced ontologies with significantly fewer categories than the complete GO ontology. The script "map2slim" (available from GO) was used to assign proteins to their nearest GO category and to identify those that are located in the extracellular or plasma membrane. Protein sequences were uploaded to the TMHMM Server v. 2.0 and the prediction algorithm was used to identify proteins expected to contain membrane spanning regions. Proteins possessing all 3 characteristics (contains a N~XS/T motif, associated with plasma membrane based on GO cellular component and contains at least one transmembrane helices by TMHMM prediction) were characterized as cell surface proteins. Proteins that lacked 1 or more of these features were also characterized as cell surface proteins if there was strong experimental evidence for a cell surface origin based on literature review.

A total of 411 cell surface proteins have been identified. **Figure 1** presents a Venn diagram of the overlap in surface proteins identified in the two cell types. These proteins fit Gene Ontology defined molecular function categories expected for membrane proteins **(Figure 2).**

Figure 1. Venn diagram of overlap between cell surface proteins identified from analysis of OVCAR3 and Serous Ovarian Cancer (SOC) cells

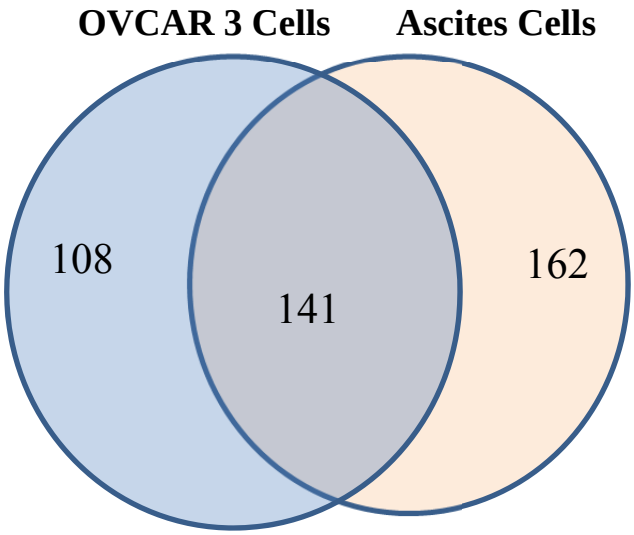
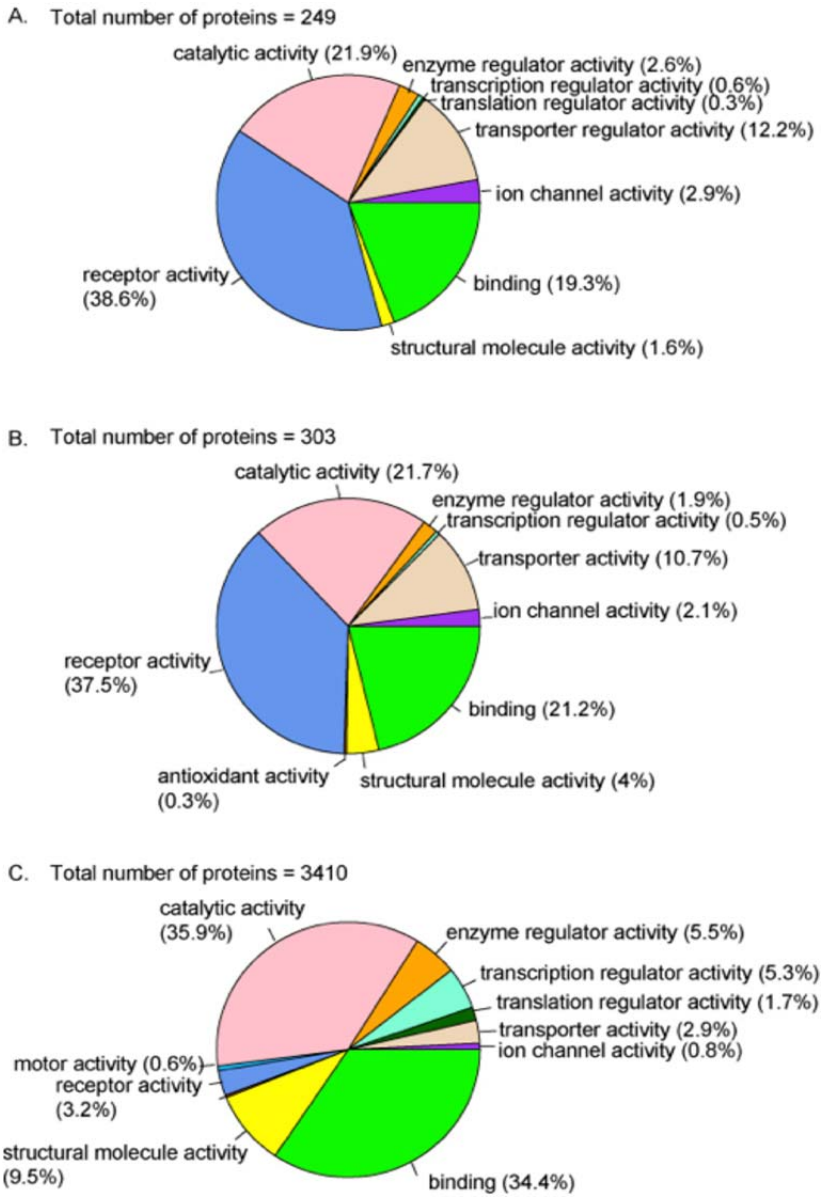


Figure 2. Molecular Function of surface proteins identified by cell surface capture (CSC) from OVCAR3 (a) and SOC cells (b) compared to proteins identified by LC-MS/MS shotgun analysis of OVCAR3 whole cell lysates (c)



To identify even more relevant targets, we have just applied this approach to normal Human Ovarian Surface Epithelial Cells (HOSE, data pending) and plan experiments with fresh fallopian tube and ovarian cancer tumor tissue in the very near future.

Polysome work:

- We have some novel splicing candidates from RNAseq data to validate.
- These novel junctions have lower reads than the normal ones.
- Polysome RNA may be able to separate the novel junctions from the novel ones.
- Because these are surface proteins, we may want to enrich these RNAs.

<u>Aim 2)</u> Prioritize ovarian cancer-associated surface proteins for their utility as molecular photoacoustic imaging targets and validate their expression in ovarian cancer tissues by immunohistochemistry. Generate high affinity scFv to 3-5 of the most promising markers.

<u>Task 5 (Months 10-12):</u> *Rank candidate ovarian surface proteins through integration with other dataset*

1. Gene Ontology for cellular location
2. Proteins identified from profiling numerous sources of benign and normal tissue and plasma
3. Transcriptomic dataset derived for 35 normal tissues

In order to prioritize cell surface proteins as candidate targets for molecular imaging probes the surface protein dataset was integrated with several comprehensive protein and gene expression databases available to our group including a) gene expression data in serous ovarian cancer relative to normal ovary and fallopian tube (kindly provided by PO Brown; data generated using Stanford HEEBO arrays containing 44,544 70-mer probes and printed at Stanford Functional Genomics Facility. Details on the arrays and the protocols used are available on

the Stanford Functional Genomics website (http://www.microarray.org/sfgf/) b) 889 confident plasma proteins identified by the Plasma Proteome Project, (c) normal plasma proteins as derived from a list of approximately 4900 IPIs identified in the plasma of cancer-free women (derived from over 1,000 MS/MS interrogations of highly fractionated plasma), (d) over 3,500 proteins identified in cancer ascites fluid (a fluid proximal to a tumor), (e) transcript signatures derived from a normal human tissue expression data set including 36 types of normal human tissues [1] and f) data on normal tissue protein expression available from The Human Protein Atlas website (www.proteinatlas.org). The Human Protein Atlas project provides a comprehensive antibody-based expression and localization profile for over 10,000 human protein coding genes in 66 cell types from 48 normal human tissues and in 20 cancer types [2].

Cell surface proteins were prioritized based on the following criteria 1) highly abundant on the surface of OVCAR3 or SOCs based on spectral count 2) over-expressed in serous ovarian cancer tissue relative to normal ovary and/or fallopian tube tissue based on transcript arrays 3) lowly abundant or absent in plasma and ascites fluid and 4) low or absent expression in normal somatic tissues based on gene expression arrays and public data available on The Human Protein Atlas website. The normal tissue gene expression profile dataset was modified and processed as described by Fang [3] and mean intensities and standard deviation of expression level across all normal tissues was determined for each of 12218 genes. For each gene we identified tissues with "high expression" of that gene defined as an expression level exceeded 1 standard deviation above the median level for all genes in all tissues.

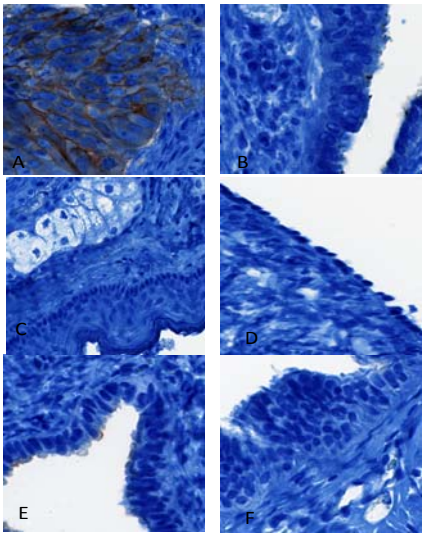
Task 6 (Months 12-24): *Validate high ranking markers in ovarian cancer tissue*

1. Establish the assay conditions and screen up to 15 markers by IHC in ovarian cancer TMAs
2. Perform co-localization studies as necessary to confirm epithelial membrane location for the most promising markers.
3. IHC analysis of the most promising markers in occult cancer tissue samples

Table 1 lists six candidate targets for molecular imaging probes, including associated annotation. Targets were also selected based on the availability of commercial antibodies suitable for immunohistochemistry (IHC). IHC confirmed cell surface expression in human serous ovarian cancers for 4 candidates tested (FOLR1, MGFE8, ITGB6 and TACSTD2) (Data not shown). We were unable to validate predicted cell surface expression for ST14 and CSPG5 using available antibodies. We are continuing to test antibodies and optimize conditions for IHC of these markers. Further studies using tissue from benign ovarian tumors and normal ovarian and fallopian tube demonstrate cancer specific expression for FOLR1 (**Figure 3**).

Table 1. Candidate targets for ovarian cancer molecular imaging probes.

Gene symbol	FOLR1	MFGE8	ITGB8	TACSTD2	CSPG5	ST14
Contains N~XS/T motif	Y	Y	Y	Y	Y	Y
Cell surface localization by GO	Y	N	Y	Y	Y	Y
Membrane Helix by TMHMM	Y	N	Y	Y	Y	Y
Relative expression in SOC tissue vs. normal ovary (mRNA)	3.07	0.46	1.89	2.83	2.05	2.6
Relative expression in SOC tissue vs. normal fallopian tube (mRNA)	0.98	1.07	2.23	1.36	0.47	0.99
Detected in plasma from healthy women by LC-MS/MS	Not detected	Y	Not detected	Not detected	Not detected	Not detected
Normal tissues with "high expression"* (mRNA)	Salivary. Lung, Trachea	Heart, Ovary, Prostate, Trachea, Bladder, Uterus	Pancreas, Ovary Breast, Kidney, Bone marrow	Skin; Trachea, Breast, Salivary	Brain, Spinal Cord	Colon, Intestine, Salivary, Trachea, Uterus
Number of normal tissue types demonstrating protein expression as reported by The Human Protein Atlas	Not reported	13 out of 64	35 out of 66	Not reported	Not reported	Not reported



Additional IHC studies confirm that FOLR1 is expressed on surface a majority of serous ovarian cancer tissues (8 of 10 tissues tested to date) but is not expressed in borderline or benign ovarian tumors or normal ovary and fallopian tubes (**Figure 3**). This cancer selective expression profile makes FOLR1 an ideal target for specific imaging of early ovarian cancer. Additional studies are underway to evaluate FOLR1 expression in hundreds of ovarian cancers and 66 normal tissue types by TMA and in high-grade early stage serous cancer and occult cancers identified at RRSO.

Figure 3. FOLR1 expression in normal fallopian tube and normal and benign ovarian tissue compared to serous ovarian cancer. A) High grade invasive serous cancer b) Serous borderline tumor c) benign dermoid d) normal ovary e) normal pre-menopausal FT and) normal post-menopausal FT. Representative images from 1 of 5 tissues stained.

Task 7 (Months 18-24): Develop high affinity scFv against 3-5 of the most promising markers based on earlier studies

1. Express antigen in mammalian system
2. Iteratively screen yeast scFv libraries and identify high affinity scFv
3. Confirm specificity of scFv by western blot, FACS analysis and measuring K_d

We are currently refining our list of candidates to identify the most appropriate markers for antibody development.

KEY RESEARCH ACCOMPLISHMENTS

1) Using the N-glycoprotein cell surface capture method we have identified 411 ovarian cancer cell surface proteins
2) We have annotated all of these proteins and identified several candidate targets for ovarian cancer molecular imaging probes that are in various stages of validation
3) We have identified FOLR1 as a highly promising target for photoacoustic molecular imaging of ovarian cancer

REPORTABLE OUTCOMES

Accomplishments 1-3 above are described in a nearly complete manuscript that will be submitted in the next 1-2 weeks.

CONCLUSION

FOLR1 is a highly promising target for photoacoustic molecular imaging of ovarian cancer. We are proposing to translate this finding to women in a project in the upcoming POCRC SPORE competing renewal application due in 2013.

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